creative commons
COMMONS DEED

저작자표시-비영리-변경금지 2.0 대한민국

이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

이학석사 학위논문

알파시뉴클레인 응집체의 세포 내 유입을 조절하는 새로운 막단백질의 분리

Isolation of Novel Membrane Proteins Mediating Cellular Uptake of Oligomeric α-Synuclein

2019 년 8 월

서울대학교 대학원

생명과학부

이 하 늘

알파시뉴클레인 응집체의 세포 내 유입을 조절하는 새로운 막단백질의 분리

Isolation of Novel Membrane Proteins Mediating Cellular Uptake of Oligomeric α-Synuclein

지도 교수 정 용 근

이 논문을 이학석사 학위논문으로 제출함
2019 년 8 월

서울대학교 대학원
생명과학부
이 하 늘

이하늘의 이학석사 학위논문을 인준함
2019 년 8 월

위 원 장 박 동 은 (인)

부위원장 정 용 근 (인)

위 원 박 상 현 (인)

ABSTRACT

Isolation of Novel Membrane Proteins Mediating Oligomeric α-Synuclein Uptake

Haneul Lee

School of Biological Sciences

The Graduate School

Seoul National University

Parkinson's disease (PD) is a neurodegenerative movement disorder and is characterized neuropathologically by the accumulation of α-synuclein protein aggregates. α-Synuclein may contribute to PD pathogenesis in various ways. Emerging evidence suggests that α-synuclein can be transmitted and propagate between neurons and can seed the formation of toxic aggregates in recipient neurons in a prion-like manner. However, the mechanism of cell-to-cell transmission of α-synuclein

aggregates is poorly understood. In a recent study, the cell-surface lymphocyte activation gene 3 protein (LAG3/CD233) was reported as a neuronal receptor mediating the endocytosis of α-synuclein pre-formed fibrils (PFF). Additionally, α-synuclein forms multiple kinds of species and might bind to several other receptors at the plasma membrane. Here, I established and utilized a cell-based assay to isolate the membrane proteins that could mediate cellular uptake and cell-to-cell propagation of α-synuclein. α-Synuclein was expressed in *E. coli,* purified and incubated *in vitro* to form oligomers. In addition, I prepared cDNA expression libraries encoding membrane proteins. With these cell-based assays, I functionally screened a cDNA expression library enriched in human genes encoding transmembrane proteins. From the primary and secondary functional screening, several putative positive clones were identified. Among those positive clones, MTM6A12 was the most effective in its binding to α-synuclein. Overexpression of the MTM6A12 increased membrane binding of α-synuclein and cellular uptake of α-synuclein in a dose-dependent manner. MTM6A12 could only bind to α-synuclein, not Aβ and Tau. Thus, I have successfully isolated membrane proteins that might contribute to the propagation of α-synuclein for PD pathogenesis.

Keywords: Parkinson's disease, α-synuclein, synucleinopathy, receptors, uptake, cell-to-cell transmission, propagation

Student Number: 2017-24913

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

AAV	Adeno-associated virus
AD	Alzheimer's disease
AP	Alkaline phosphatase
Aβ	Amyloid beta
α-syn	Alpha synuclein
DMEM	Dulbecco modified Eagle medium
E. Coli	*Escherichia coli*
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FcγRIIB	Fc fragment of IgG receptor IIb
FPLC	Fast protein liquid chromatography
GPCR	G-protein coupled receptor
GSK	Glycogen synthase kinase
HSPG	Heparan sulfate proteoglycan
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
KO	Knockout
LAG3	Lymphocyte-activation gene 3
LB	Lysogeny broth

Ni-NTA	Nickel-nitrilotriacetic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFF	Pre-formed fibrils
ROS	Reactive oxygen species
S1P1	Sphingosine-1-phosphate receptor 1
SEC	Size exclusion chromatography
TLR2	Toll-like receptor 2
TM	Transmembrane

INTRODUCTION

The aggregation and accumulation of specific proteins in the brain are common neuropathological features of many neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD) and dementia with Lewy bodies (Jucker & Walker, 2013; Vaquer-Alicea & Diamond, 2019). PD is a common progressive neurodegenerative disease characterized pathologically by the loss of dopaminergic neurons in the midbrain and the presence of intraneuronal inclusions, called Lewy body, mainly consisting of aggregates of α-synuclein (Damier et al., 1999; Halliday et al., 2011). Accumulation of insoluble α-synuclein fibrils in the brain is also observed in dementia with Lewy bodies and multiple system atrophy, called synucleinopathy (McCann et al., 2014).

α-synuclein is an acidic protein with 140 amino acids and is highly expressed in neurons and enriched in presynaptic terminals. The precise physiological function of α-synuclein remains unclear, but it is thought to be involved in synaptic vesicle trafficking and synaptic plasticity (Scott et al., 2010). Dysfunction of α-synuclein is associated with idiopathic and genetically inherited PD.

Recent studies highlighted the important role of extracellular α-synuclein. Extracellular forms of α-synuclein propagate between various types of cells, bind to cell surface receptors and transmit signals, regulating numerous intracellular processes. Multiple studies suggest that extracellular α-synuclein acts as a specific ligand for cell surface receptors (Yamada & Iwatsubo, 2018). Binding of α-synuclein to cell surface receptors induced various biochemical and physiological reactions through different mechanisms (Surguchev & Emamzadeh, 2019). α-Synuclein oligomers released from neuronal cells is known to induce inflammatory responses in microglia. These responses are mediated by the activation of toll-like receptor 2 (TLR2) signaling (Kim et al., 2013). Extracellular α-synuclein-induced expulsion of S1P1 receptor impairs the inhibitory G-protein signaling (Badawy et al., 2018). The internalization of α-synuclein species by neighboring cells can be also regarded as a clearing mechanism, particularly when microglia are involved (Lee et al., 2008; Stefanova et al., 2011). Intracellular uptake of α-synuclein may be eliminated depending on the cell type, which may have a positive effect on PD pathology (Loria et al., 2017). Also, many α-synuclein-interacting intracellular proteins have been identified and transmit signals affecting intracellular processes (Breydo et al., 2012). For

example, it was reported that extracellular α-synuclein causes microtubule destabilization via GSK-3-dependent tau phosphorylation (Gąssowska et al., 2014).

Cell-to-cell spreading of α-synuclein has become an attractive model to explain the progress of Parkinson's disease and the typical patterns of pathology deposition in anatomically connected regions of the diseased brain (Goedert et al., 2010). The evidence of cell-to-cell transfer of α-synuclein inclusions had come from research showing that misfolded intraneuronal α-synuclein can transfer to neighboring cells both in culture and in transgenic mice (Desplats et al., 2009). Several studies have shown that Lewy bodies may propagate within the brain (Kordower et al., 2008; Li et al., 2008). α-Synuclein can transmit from neuron to neuron, causing propagation and neurotoxicity in a prion-like manner (Volpicelli-Daley et al., 2011; Luk et al., 2012). Recently, lymphocyte-activation gene 3 (LAG3) was reported as a receptor to mediate α-synuclein transmission (Mao et al., 2016). However, the α-synuclein transmission was not completely blocked in LAG3 null primary neuron and KO mice. Accordingly, other membrane receptors, such as FcγRIIB and HSPGs, were reported to be involved in cellular uptake of α-synuclein (Holmes et al., 2013; Choi et al., 2018). It means that various receptors engage in the

propagation according to various toxic oligomeric species and cell types, and affect PD pathology in different ways. Thus, identifying the α-synuclein receptors and exploring its role of propagation will help to understand the pathogenesis of Parkinson's disease.

Here, I established a cell-based assay to isolate novel membrane proteins regulating the cell-to-cell transmission of α-synuclein and screened a cDNA expression library enriched in human genes encoding transmembrane proteins.

MATERIALS AND METHODS

Expression and purification of α-synuclein

E. coli BL21 cells were transformed with a pET21a-LIC plasmid containing C-terminus 6x His-tagged human α-synuclein cDNA. The transformed bacteria were grown in LB medium with ampicillin (0.1 mg/ml) at 37 ℃ to an A_{600} of 0.6, at which 1 mM of IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the culture medium. After 3 h of induction, the cells were harvested at 2,000 x g for 10 min at 4 ℃. The pellet was suspended with His-binding buffer (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.1 mM EDTA,) containing 1mM phenylmethylsulfonyl fluoride (PMSF). Then, the cells were sonicated and followed by centrifugation at 13,000 x g for 10 min at 4 ℃. The supernatants were heated at 95 ℃ for 5 min and followed by centrifugation at 13,000 x g for 10 min at 4 ℃. The supernatants were filtered with a 0.45 μm filter. Ni-NTA agarose beads (Qiagen) were added and the mixture was incubated for 2 h at 4 ℃. The suspension was poured into a column and the beads were washed with His-binding buffer. The recombinant α-synuclein was eluted in elution buffer (20 mM Tris-Cl, 500 mM NaCl, 800 mM imidazole, 0.1 mM EDTA,

pH 8.0). The purified α-synuclein was transferred to 10 kDa molecular weight cutoff SnakeSkin Dialysis Tube (Thermo Scientific) and dialyzed against phosphate buffered saline (PBS). The recombinant α-synuclein concentration was determined by measuring the absorbance at 280 nm.

In vitro oligomerization of α-synuclein

The recombinant α-synuclein (100 μM) was incubated with orbital agitation (1,000 RPM at 37 ℃) for 3 days. The incubated α-synuclein was sonicated briefly and kept at −80 ℃.

Size exclusion chromatography

Monomeric α-synuclein and oligomeric α-synuclein by *in vitro* oligomerization was separated using a Superdex 200 10/300 GL column (GE Healthcare) on an AKTA FPLC system, equilibrated with PBS.

Preparation of oligomeric α-syn-biotin

The recombinant α-synuclein monomer was purified and labeled with EZ-link Sulfo-NHS-LC-Biotin Kit (Thermo Scientific) according to the manufacturer's instructions. After conjugation,

oligomeric α-syn-biotin was prepared as mentioned above.

Preparation human cDNA library

The Human cDNA library was prepared as follows. The cDNAs encoding TM domain were searched in the gene collection list of our laboratory and then purified by DNA preparation column (GeneAll) for transfection into mammalian cells.

Cell culture and DNA transfection

SH-SY5Y, HEK293T cells were cultured in DMEM (HyClone) supplemented with 10 % fetal bovine serum (FBS) (Gibco), gentamicin (Gibco), penicillin/streptomycin (Gibco). Cells were grown at 37 ℃ under an atmosphere of 5 % CO_2. Cells were transfected using Lipofector-pMAX reagent (APTABIO) according to the manufacturer's instructions.

Western blot analysis and antibodies

Cells were harvested and analyzed as described previously (Gwon et al., 2018). Cells were lysis in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 % sodium deoxycholate, 1 mM PMSF). The lysates were centrifuged at

14,000 g for 10 min at 4 ℃ and the supernatant was separated by SDS-PAGE and blotted onto PVDF membrane. The blots were blocked for 1 h at room temperature and incubated with the following antibodies: anti-α-synuclein 4D6 (Abcam), anti-His (Santa Cruz), anti-α-tubulin (Sigma-Aldrich). Membranes were rinsed three times with TBS-T (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1 % Tween-20), further incubated for 1 h with peroxidase-conjugated secondary antibodies and visualized using the ECL detection system.

Cell-based biotin assay

HEK293T cells were transfected with indicated genes for 48 h and further incubated with α-syn-biotin in DMEM (10 % FBS) media for 2 h. Unbound α-syn-biotin was removed by extensive washing with DMEM (10 % FBS). The cells were fixed with 4 % paraformaldehyde for 20 min, washed three times with TBS. Then, the cells were blocked for 30 min with 10 % FBS serum and 0.1 % Triton X-100 in TBS and incubated at 4 ℃ for 16 h with alkaline phosphatase (AP) conjugated streptavidin (1:2000, Sigma-Aldrich) in TBS supplemented with 5 % FBS serum and 0.05 % Triton X-100. Finally, unbound streptavidin-AP was washed three times with TBS and bound streptavidin-AP was visualized by 5-bromo-4-

chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich) reaction. The reaction was stopped with water after 10 min. Quantification of bound α-syn-biotin to the cells was performed with ImageJ softwere.

Z-stack imaging

Confocal imaging was performed using LSM700 (Carl Zeiss). Z-stacks were generated from images taken at 0.47 μm intervals. Zen software was used to analyze Z-stack.

ImageJ analysis

A threshold was selected under Image/Adjust to achieve a desired range of intensity values for each experiment. Once determined, this threshold was applied to all the images in each experiment. The threshold setting was also used to exclude the background. After the exclusion of the background, the selected area in the signal intensity range of the threshold was measured using the measurement option under the Analyze/Measure menu. The area values with different concentrations of α-syn-biotin were input into the Prism program to obtain K_d value.

RESULTS

Establishment of a cell-based gain of function genetic screening assay.

To identify novel α-synuclein-binding membrane proteins, I designed a cell-based gain of function genetic screening assay (Figure 1). In brief, I introduced each of the human cDNA expression plasmids onto HEK293T cells that were grown in 96 well culture plate. Two days after transfection, the cells were briefly incubated for 2 h with oligomeric α-synuclein that was labeled with biotin (α-syn-biotin, 1 μM). α-Syn-biotin was then washed and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The α-syn-biotin bound to the cells was incubated with streptavidin-AP and washed for observation under a microscope.

His tagged α-synuclein was expressed in *E. coli* BL21 and purified with affinity chromatography using Ni-NTA resin. For sample preparation before chromatography, cell lysate was heated to remove heat unstable bacterial proteins (Pujols et al., 2017). Purified α-synuclein was biotinylated and analyzed with Coomassie Blue staining for the purity (Figure 2A) and with

immunoblot assay for biotinylation (Figure 2B). To generate oligomeric α-synuclein, monomeric α-synuclein was diluted at 100 μM, incubated with orbital agitation (1,000 RPM at 37 ℃) for 3 days and sonicated. The sample was then separated by FPLC using a Superdex 200 increase 10/300 GL column. The monomeric α-synuclein was eluted in a peak corresponding to a column volume of 13~15 ml (Figure 2C), while oligomeric α-synuclein was eluted in a peak corresponding to a column volume of approximately 9 ml (Figure 2C). Preparation of oligomeric α-synuclein was kept at −80 ℃ until use.

The expression cDNA libraries were prepared as follows. First, the list of 17 genes, which were reported to be genetically and sporadically related to Parkinson's disease by many groups, were collected, named as PD-associated genes (Brás et al., 2015). Second, 222 CCSB-Broad Lentiviral Expression GPCRs, a commercially available cDNA library from Dharmacon Inc., was prepared. Third, 1,500 cDNAs encoding single-transmembrane (TM) and Multi-TM were collected (Figure 3A). The cDNA libraries were purified for cell transfection and the quality of cDNA plasmids was examined by DNA agarose gel electrophoresis (Figure 3B).

Using this preparation, the cell-based α-synuclein biotin

assays on multi-well culture plate were performed and confirmed, showing that α-syn-biotin binds to cells in a dose-dependent manner under the screening condition (Figure 4A and B). As a positive control, I used APLP1 that was reported to bind to α-synuclein (Mao et al., 2016).

Functional screening and isolation of α-synuclein binding membrane proteins.

Next, I performed the screening to isolate α-synuclein binding membrane proteins using the cell-based assay and cDNA expression library. In the primary screening, HEK293T cells were transiently transfected with the human cDNA libraries and incubated with α-syn-biotin. Cell images after streptoavidin reaction were captured with IN Cell Analyzer 2000 (GE Healthcare Inc.) and the area of α-synuclein bound to cells was quantified (Figure 5). From the primary screening of 1,800 cDNAs, including PD-associated genes (Figure 5A), GPCR (Figure 5B) and membrane proteins (Figure 5C), the putative positive clones showing high score comparable to that of the positive control were isolated.

These putative positive clones were tested again with the same

assay for their activities, the secondary screening. A total of 36 putative positive clones from membrane proteins were tested again using the same way (Figure 6A and B). Finally, from the secondary screening and considering their cellular localization, tissue expression, and neuronal function, four positive clones, such as PD1, STM1D9, STM2A5 and MTM6A12, were isolated. These clones were assessed again for their ability to bind to α-synuclein, showing that the clones bind to α-synuclein in a dose-dependent manner (Figure 7 A and B). Most of the positive clones could bind to α-synuclein. The information of the positive clones is briefly described (Figure 7C).

MTM6A12 binds to oligomeric α-synuclein and mediates the cellular uptake of oligomeric α-synuclein.

Among these positive clones, MTM6A12 was the most effective in its ability to bind to α-synuclein. Thus, I characterized the role of MTM6A12 in α-synuclein biology and pathology. Determination of binding affinity using cell-based assay revealed that overexpressed MTM6A12 in cells binds to α-synuclein with K_d value 396.4 nM (Figure 8A and B). In addition, I tested whether MTM6A12 plays a

role in the cellular uptake of α-synuclein (Figure 9). When SH-SY5Y cells overexpressing MTM6A12 were incubated with oligomeric α-synuclein, the results from immunoblotting of whole cell lysate revealed that overexpressed MTM6A12 increased cellular uptake of α-synuclein. Under this condition, the α-synuclein level in the culture medium was reduced (Figure 9A), showing that α-synuclein level inside cell inversely correlates to that of α-synuclein in the culture medium.

In addition, I assessed cellular uptake of α-synuclein using a fluorescence-labeled, purified α-synuclein protein. SH-SH5Y cells overexpressing MTM6A12 were treated with oligomeric α-synuclein that was labeled with Dylight488 dye. After 2 h, cells were washed with PBS, fixed and then observed under a fluorescence microscope (Figure 9B). To assure cellular internalization of α-synuclein-Dylight488, SH-SY5Y cells were transfected with MTM6A12 fused with red-fluorescence protein (RFP) at its C-terminus. With confocal microscopy, Z-stack imaging analysis of these cells expressing MTM6A12-RFP confirmed the presence of internalized α-synuclein inside the cells (Figure 9C).

Next, I performed the cell-based biotin assay to address the binding specificity of MTM6A12 to Aβ and Tau. Oligomeric forms

of Aβ and Tau that are aggregate proteins associated with Alzheimer's disease were prepared. FcγRIIb that is known to bind to Aβ (Kam et al., 2013; Gwon et al., 2018), and TauR that was isolated to bind to tau in this lab but not published yet (Kim in preparation 2019), were used as a positive control in binding to Aβ and Tau, respectively (Figure 10A and C). The results of cell-based binding assays revealed that MTM6A12 could bind only to oligomeric α-synuclein, not Aβ and Tau (Figure 10B and D), indicating that MTM6A12 selectively binds to α-synuclein.

MTM6A12 mediates the cell-to-cell transfer of α-synuclein.

To monitor whether or not MTM6A12 plays a role in the cell-to-cell transfer of α-synuclein, I used an adeno-associated viral (AAV) vector (α-syn-DsRed-IRES-EGFP) harboring both α-synuclein fused to an RFP (α-syn-DsRed) and EGFP that is to label donor cells. HEK293T cells were transfected with α-syn-DsRed-IRES-EGFP or ER-DsRed-IRES-EGFP control construct (Figure 11A). The donor cells directly transfected with and thus expressing both α-syn-DsRed and EGFP are yellow under a fluorescence microscope. If α-synuclein is transferred from the

donor cells to the surrounding cells, recipient cells then exhibit only RFP signal of α-syn-DsRed but not GFP signal, thereby demonstrating the cell-to-cell transfer of α-synuclein (Figure 11 B and C). When I tested the cell-to-cell transfer of α-synuclein using these assays, ectopic expression of MTM6A12 increased the ratio of RFP to GFP signal compared to control (Figure 11D and E). These results suggest that MTM6A12 might contribute to the cell-to-cell transfer of α-synuclein.

Figure 1. A schematic diagram of the cell-based α-synuclein biotin assay.

Schematic diagram outlining the strategy for the screening of oligomeric α-syn-biotin-binding membrane proteins. HEK293T cells were transfected with cDNA expression libraries enriched in human genes encoding transmembrane proteins. After transfection for 48 h, cells were incubated with oligomeric α-syn-biotin (1,000 nM α-syn-biotin monomer-equivalent concentration) for 2 h and with alkaline-phosphatase-conjugated streptavidin (Dilution 1: 2,000) for 16 h. Then, the bound streptavidin-AP was visualized by BCIP/NBT reaction.

Figure 1.

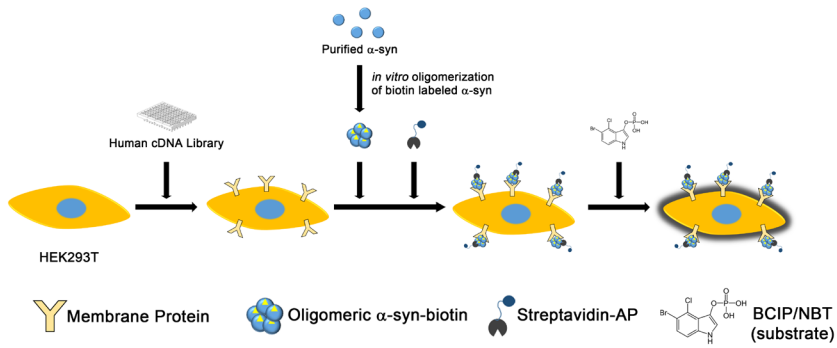
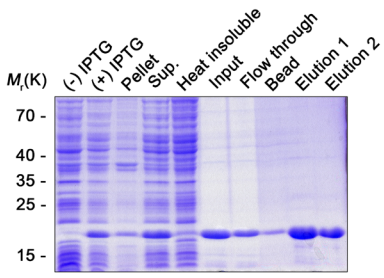

Purified α-syn
in vitro oligomerization
of biotin labeled α-syn
Human cDNA Library
HEK293T
Membrane Protein
Oligomeric α-syn-biotin
Streptavidin-AP
BCIP/NBT
(substrate)

Figure 2. Preparation and characterization of a biotinylated α-synuclein oligomer.

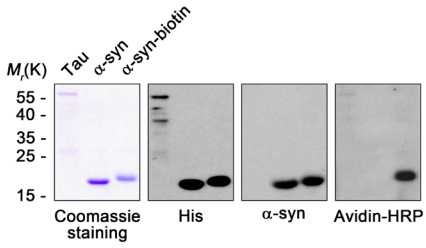
(A) Expression and purification of the recombinant α-synuclein protein. Recombinant full-length human α-synuclein was expressed in *E. coli* BL21 cells and purified using Ni-NTA agarose resin. The purified protein was analyzed by Coomassie Blue staining. (B) The recombinant α-synuclein monomer was labeled with Sulfo-NHS-LC-Biotin and resulting α-syn-biotin was validated by Coomassie Blue staining and immunoblot analysis using anti-his and anti-α-synuclein antibodies. Biotinylated α-syn was visualized by ExtrAvidin-HRP. (C) α-syn-biotin labeled α-synuclein monomer and oligomers were analyzed with size exclusion chromatography (SEC) by monitoring the peak absorbance at 280 nm. Peak 'a' and 'b' represent monomeric and oligomeric α-synucleins, respectively.

Figure 2.

A


M_r(K)
(-) IPTG
(+) IPTG
Pellet
Sup.
Heat insoluble
Input
Flow through
Bead
Elution 1
Elution 2
70 -
40 -
35 -
25 -
15 -

B


M_r(K)
Tau
α-syn
α-syn-biotin
55 -
40 -
35 -
25 -
15 -
Coomassie staining
His
α-syn
Avidin-HRP

C

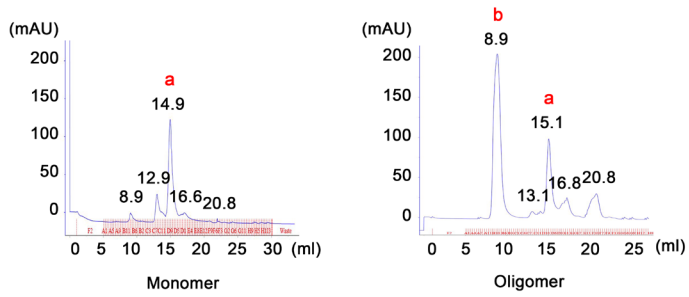

(mAU)
200
150
100
50
0
a
14.9
12.9
8.9
16.6
20.8
0 5 10 15 20 25 30 (ml)
Monomer
(mAU)
200
150
100
50
0
b
8.9
a
15.1
13.1
16.8
20.8
0 5 10 15 20 25 (ml)
Oligomer

Figure 3. Preparation of human cDNA expression library.

(A) Table of human cDNA expression library. (B) Agarose gel electrophoresis (1% agarose) showing the purified human cDNA library plasmids. 'M' denotes 1 kb DNA size marker.

Figure 3.

A

Human cDNA Library	The Number of Clones	Description
PD associated genes	17	Associated with the development of PD
GPCR	222	Human G-protein coupled receptors
Single-TM	497	Single-pass transmembrane proteins
Multi-TM	1039	Multi-pass transmembrane proteins

B

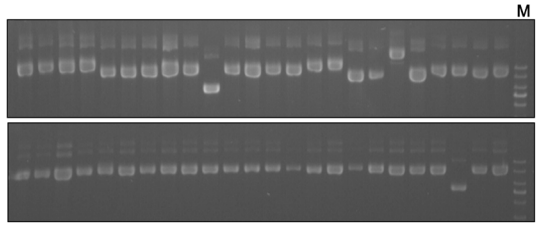
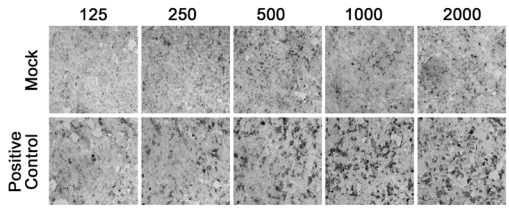


Figure 4. Establishment a cell-based assay to isolate oligomeric α-synuclein-binding membrane proteins.

(A) HEK293T cells were transfected with pcDNA (Mock) or the indicated gene for 48 h and then incubated with the indicated concentrations (125, 250, 500, 1,000, 2,000 nM) of purified oligomeric α-syn-biotin for 2 h. Cells were fixed with 4 % paraformaldehyde and incubated with streptavidin-AP (1: 2,000) for 16 h. The bound streptavidin-AP to cells was visualized by BCIP/NBT reaction. Images were captured under the Bright-field microscope. (B) Quantification of the α-syn-biotin bound to cells was performed using ImageJ software.

Figure 4.

A


125
250
500
1000
2000
Mock
Positive Control

B

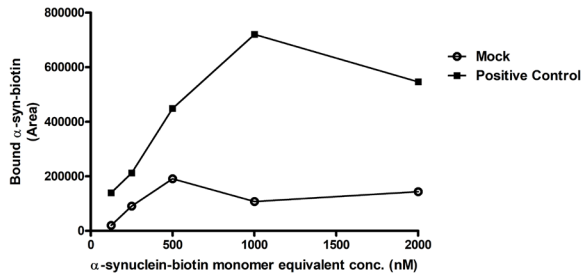

800000
600000
400000
200000
0
Bound α-syn-biotin (Area)
0
500
1000
1500
2000
α-synuclein-biotin monomer equivalent conc. (nM)
Mock
Positive Control

Figure 5. Primary screening of α-synuclein-binding membrane proteins with cell-based assays.

(A~C) Primary screening. The results of the primary screening (A). HEK293T cells were subcultured onto a 96-well culture plate a day before. Cells were transfected for 48 h with each of human cDNA expression libraries encoding GPCRs (B) or membrane proteins containing single TM (C) and further incubated with oligomeric α-syn-biotin (1,000 nM) for 2 h. Images were then captured by IN Cell Analyzer 2000 (GE Healthcare) magnified by 100X. Quantification of α-syn-biotin bound to cells expressing the clones was performed using InCell Developer Toolbox. Bars represent mean ± SEM (n = 3).

Figure 5.

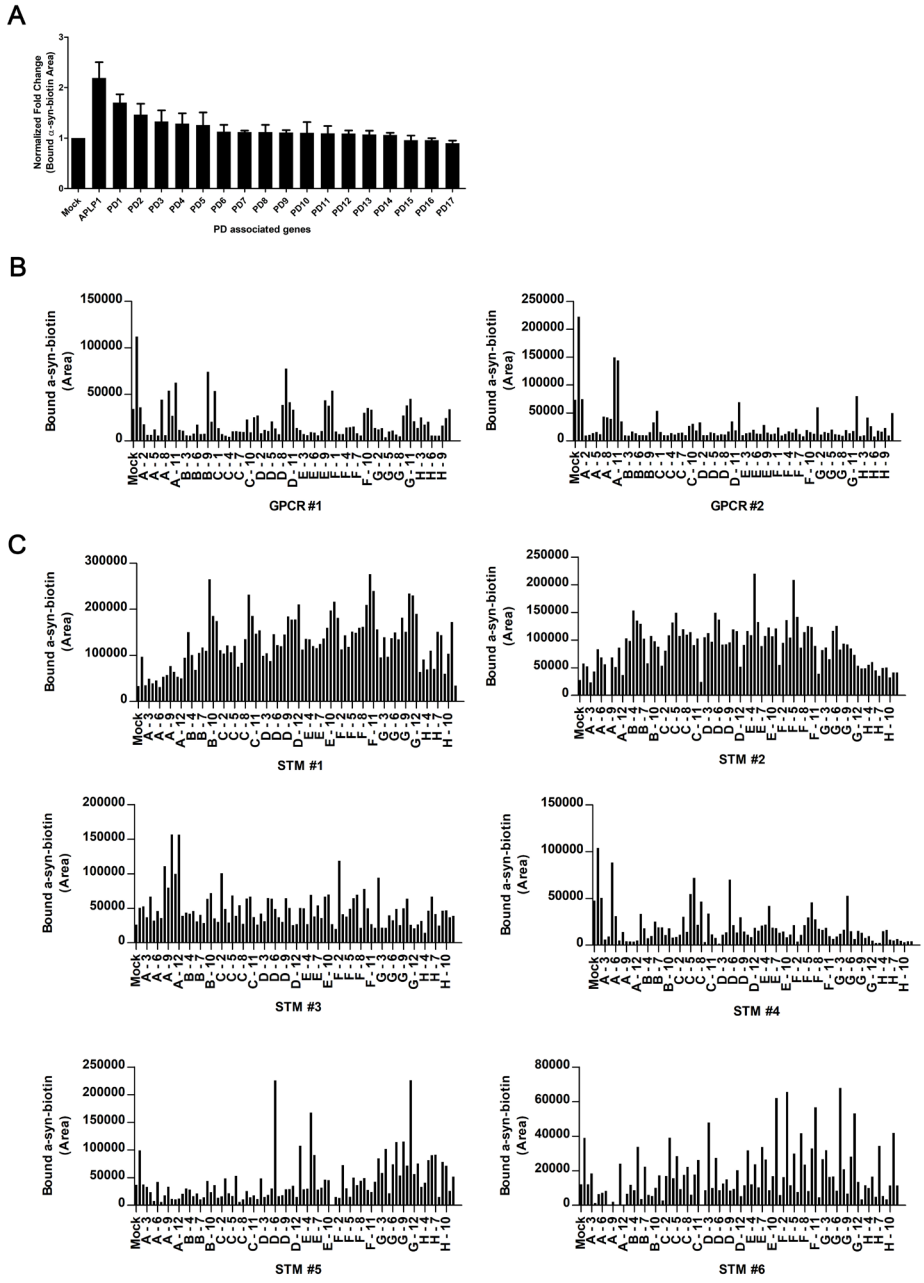
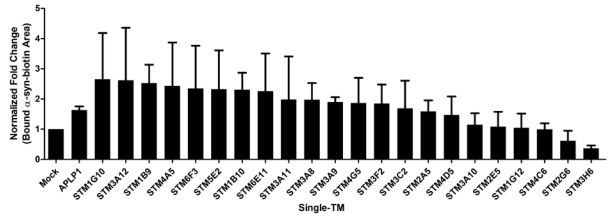

A
Normalized Fold Change (Bound α-syn-biotin Area)
Mock
ATXN1
PD1
PD2
PD3
PD4
PD5
PD6
PD7
PD8
PD9
PD10
PD11
PD12
PD13
PD14
PD15
PD16
PD17
PD associated genes
B
Bound a-syn-biotin (Area)
GPCR #1
GPCR #2
C
STM #1
STM #2
STM #3
STM #4
STM #5
STM #6

Figure 6. Secondary screening of α-synuclein-binding membrane proteins.

(A, B) Secondary screening. Results of the secondary screening of membrane proteins harboring single TM (A) or multi-TM (B). HEK293T cells were subcultured onto a 96-well culture plate a day before. Cells were transfected with the putative positive clones for 48 h and further incubated with oligomeric α-syn-biotin (1,000 nM) for 2 h. Images were then captured under Bright-field microscope and quantification of α-syn-biotin bound to cells was performed using ImageJ software. Bars represent mean ± SEM (n = 3).

Figure 6.

A



B

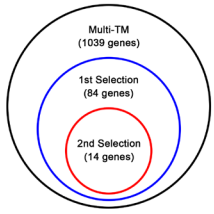


Plate	1st selection	Plate	1st selection
#1	3	#7	8
#2	11	#8	7
#3	7	#9	14
#4	3	#10	8
#5	3	#11	8
#6	4	#12	8
1st selection		84	

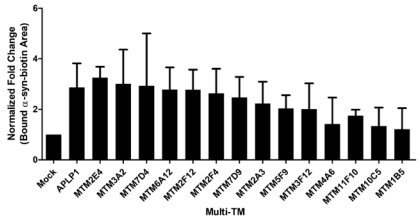
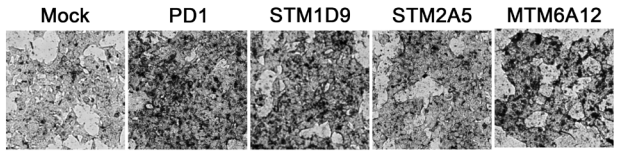


Figure 7. The putative positive clones isolated from the secondary screening.

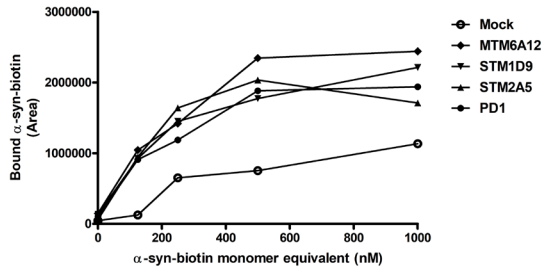
(A, B) Images of cell-based assay showing the binding of oligomeric α-syn-biotin to the clones. The putative positive clones from secondary screening were tested again. Images were captured by microscope with magnified by 100X. HEK293T cells were transfected with the putative positive clones isolated from the secondary screening for 48 h and incubated for 2 h with 1,000 nM oligomeric α-syn-biotin (A) or the indicated concentrations of oligomeric α-syn-biotin (125, 250, 500, 1000 nM) (B). Quantification of α-syn-biotin bound to cells was performed using ImageJ software. (C) List and function of the final positive clones.

Figure 7.

A



B



C

Positive Clones	Human cDNA Library	Cellular Localization	Function
PD1	PD associated genes	Plasma membrane, Cytosol	Processing of vesicular trafficking and exocytosis
STM1G10	Single-TM	Plasma membrane	Regulation of dendritic cell function
STM2A5	Single-TM	Plasma membrane	Regulation of cell adhesion
MTM6A12	Multi-TM	Plasma membrane, Nucleus	Regulation of notch signaling

Figure 8. The binding affinity of oligomeric α-synuclein to MTM6A12 in cells.

(A) HEK293T cells were transfected with pcDNA (Mock) or MTM6A12 for 24 h and incubated with oligomeric α-syn-biotin (125, 250, 500, 1,000, 2,000, 4,000 nM) for 2 h. Cells were fixed with 4 % paraformaldehyde and incubated with streptavidin-AP (1:2000) for 16 h. Streptavidin-AP bound to cells was visualized after BCIP/NBT reaction. The reaction was stopped by adding water after 10 min. Images were captured under a microscope (100X). (B) Quantification of Aβ-biotin bound to cells expressing MTM6A was performed using ImageJ softwere. K_d values are mean ± SEM and are based on monomer-equivalent concentrations

Figure 8.

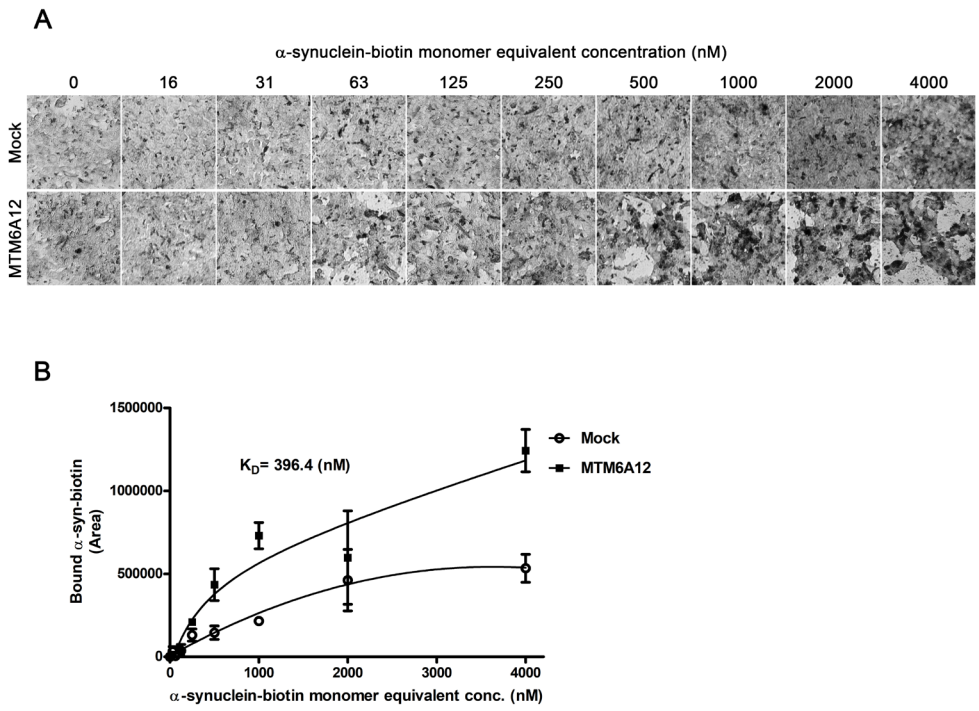

A
α-synuclein-biotin monomer equivalent concentration (nM)
0
16
31
63
125
250
500
1000
2000
4000
Mock
MTM6A12
B
Bound α-syn-biotin (Area)
1500000
1000000
500000
0
K_D= 396.4 (nM)
Mock
MTM6A12
0
1000
2000
3000
4000
α-synuclein-biotin monomer equivalent conc. (nM)

Figure 9. Overexpressed MTM6A12 enhances cellular uptake of oligomeric α-synuclein.

(A) SH-SY5Y cells were transfected with pcDNA (Mock) or MTM6A12 for 24 h and incubated with the indicated concentrations of α-synuclein oligomer for 2 h. Cells were then washed with trypsin/EDTA, and cell extracts (WCL) and culture media were analyzed by immunoblotting. (B) SH-SY5Y cells were transfected with pcDNA (Mock) or MTM6A12 for 24 h and incubated with 200 nM Dylight 488-labeled recombinant α-synuclein (green) for 2 h. Cells were then stained with DAPI (blue) and observed under a fluorescence microscope. (C) SH-SY5Y cells were transfected with MTM6A12-RFP (red) for 24 h and incubated with 500 nM Dylight 488-labeled recombinant α-synuclein (green) for 2 h. Cells were then stained with DAPI (blue) and observed under a confocal microscope.

Figure 9.

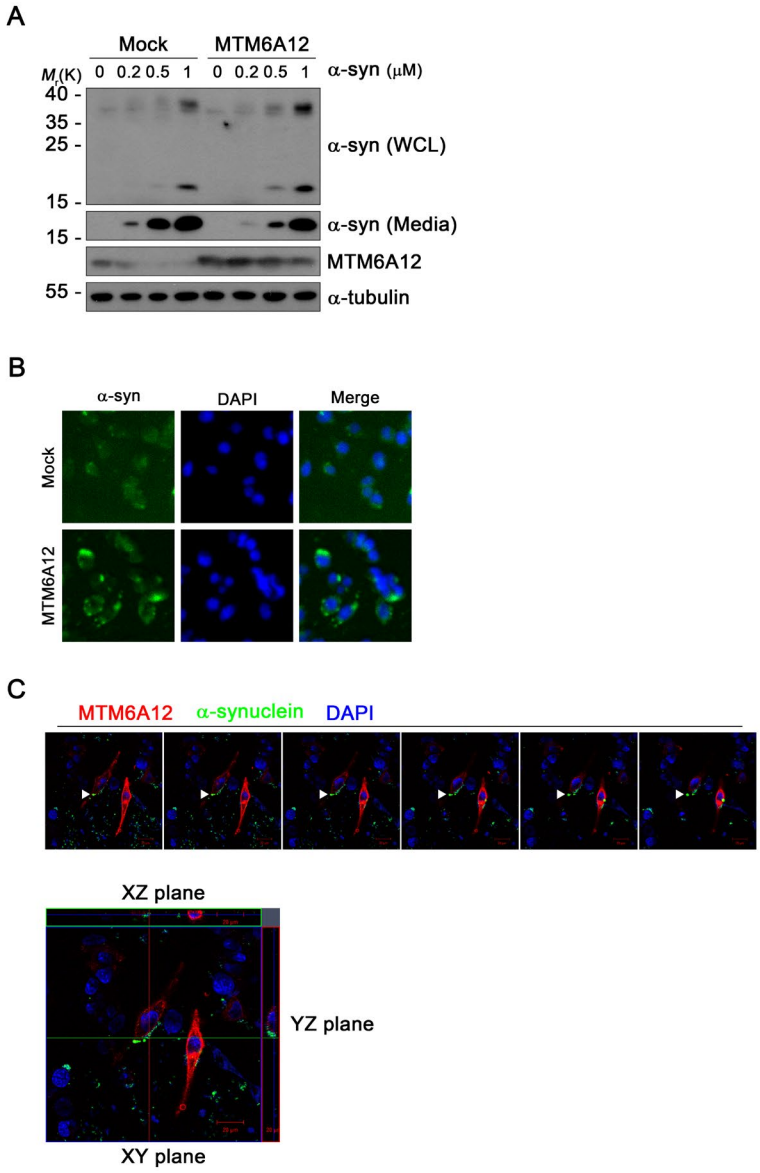

A
Mock
MTM6A12
M_r(K) 0 0.2 0.5 1 0 0.2 0.5 1 α-syn (μM)
40 -
35 -
25 -
α-syn (WCL)
15 -
15 -
α-syn (Media)
MTM6A12
55 -
α-tubulin
B
α-syn
DAPI
Merge
Mock
MTM6A12
C
MTM6A12
α-synuclein
DAPI
XZ plane
YZ plane
XY plane

Figure 10. MTM6A12 does not bind to Aβ and Tau oligomers.

(A, C) EK293T cells were transfected with pcDNA (Mock), FcgRIIb, TauR or MTM6A12 for 48 h, and incubated with biotinylated Aβ oligomer (A) or tau oligomers (C) for 2 h. Cells were then subjected to biotin assays and then observed under Bright-field microscope. FcγRIIb and TauR were used as positive controls. Images were captured with magnitude 100X. (B, D) Quantification of oligomeric Aβ (B) and tau (B) bound to cells was performed using ImageJ software. Bars represent mean ± SEM (n = 3).

Figure 10.

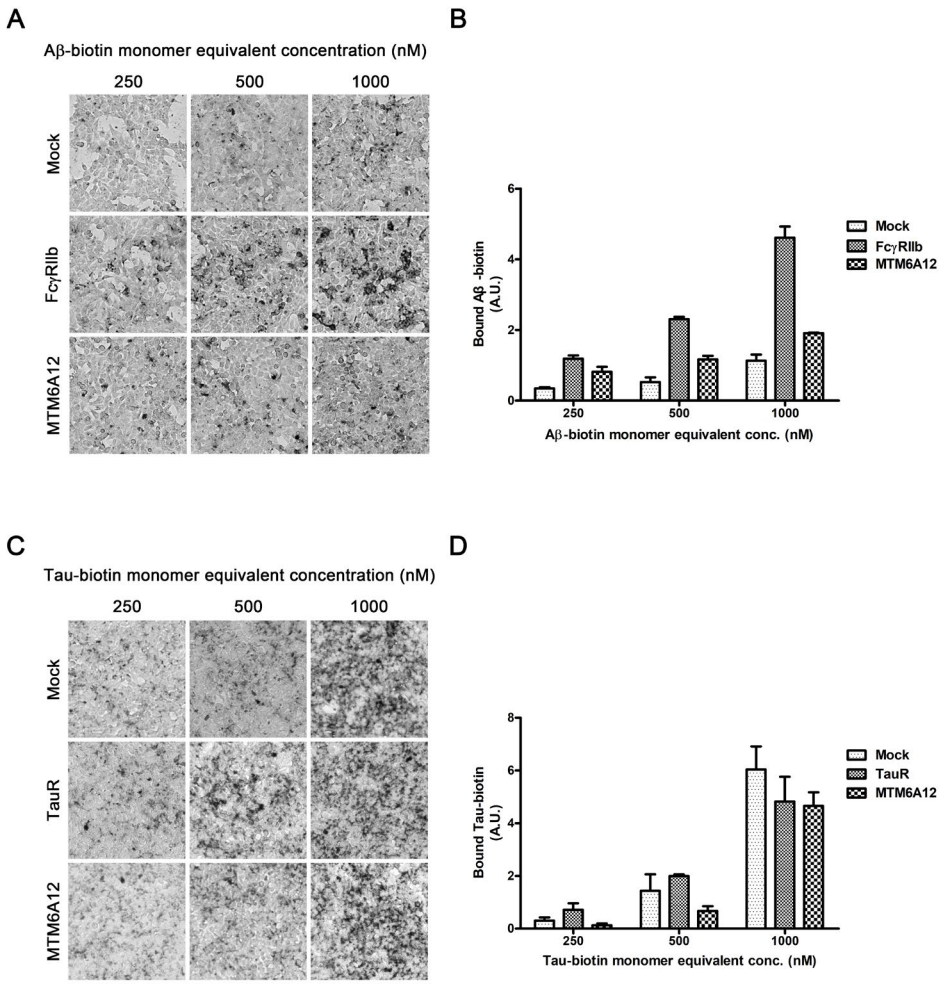
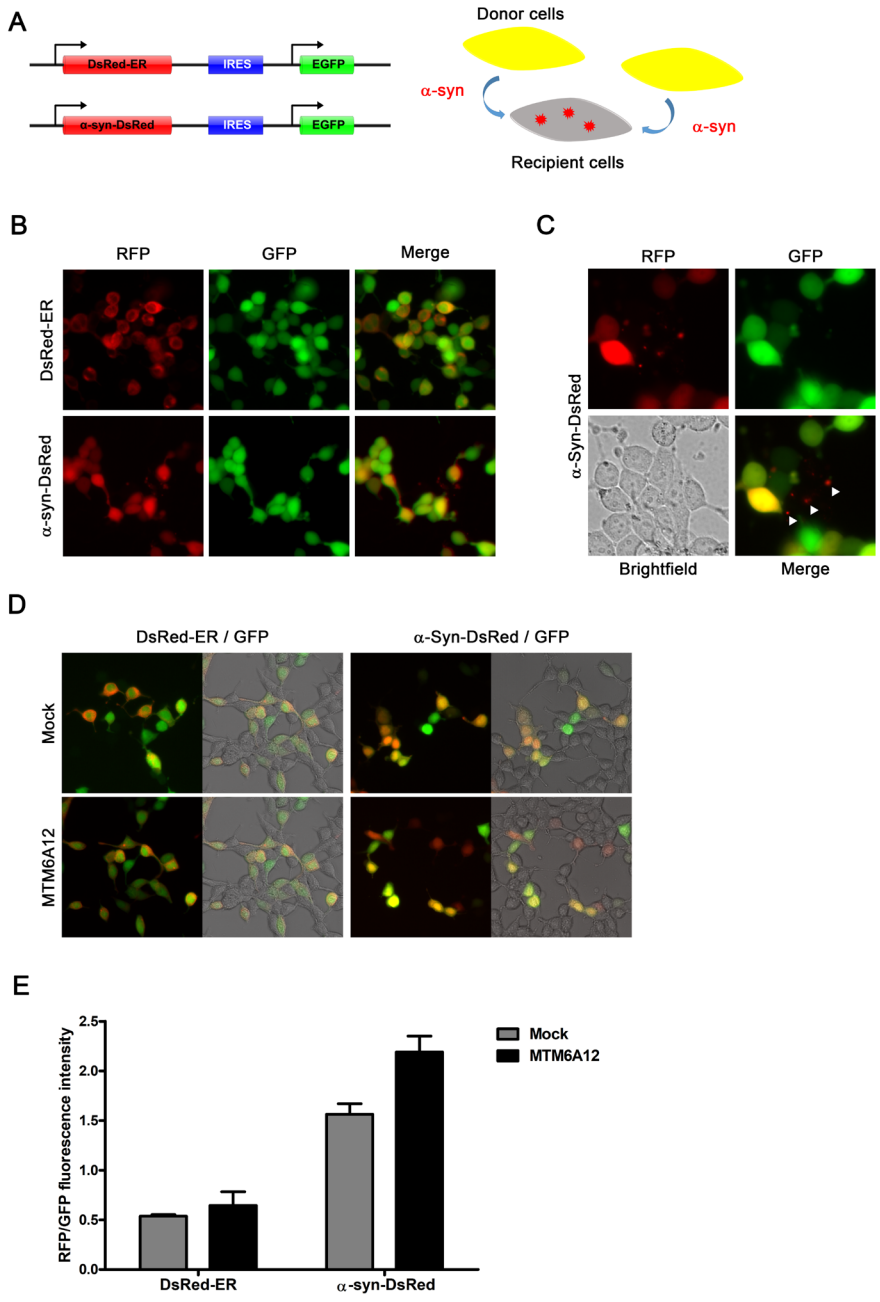

A
Aβ-biotin monomer equivalent concentration (nM)
250
500
1000
Mock
FcγRIIb
MTM6A12
B
Bound Aβ -biotin (A.U.)
Mock
FcγRIIb
MTM6A12
Aβ-biotin monomer equivalent conc. (nM)
C
Tau-biotin monomer equivalent concentration (nM)
250
500
1000
Mock
TauR
MTM6A12
D
Bound Tau-biotin (A.U.)
Mock
TauR
MTM6A12
Tau-biotin monomer equivalent conc. (nM)

Figure 11. MTM6A12 enhances the cell-to-cell transfer of α-synuclein.

(A) A schematic diagram showing DNA constructs to monitor the cell-to-cell transfer of α-synuclein. α-syn-DsRed: α-synuclein-DsRed fusion protein, IRES: internal ribosome entry site. Arrows: gene expression (left). Donor cells expressing α-syn-DsRed and EGFP exhibit yellow and recipient cells showing red fluorescence (α-syn-DsRed) harbor cell-to-cell propagated α-syn-DsRed (right). (B, C) HEK293T cells were transfected with DsRed-ER/EGFP or α-syn-DsRed/EGFP for 48 h and observed under a fluorescence microscope. Most of the cells are yellow with α-syn-DsRed/EGFP image (B) but a few cells are red with dots of α-syn-DsRed image (C, arrowheads). (D, E) HEK293T cells were co-transfected with DsRed-ER/EGFP or α-syn-DsRed/EGFP together with MTM6A12 for 48 h and then observed under a fluorescence microscope (D). The images were quantified for the relative ratio of RFP to GFP signal (E). Bars represent mean ± SEM (n = 3).

Figure 11.


A
DsRed-ER
IRES
EGFP
α-syn-DsRed
IRES
EGFP
Donor cells
α-syn
α-syn
Recipient cells
B
RFP
GFP
Merge
DsRed-ER
α-syn-DsRed
C
RFP
GFP
α-Syn-DsRed
Brightfield
Merge
D
DsRed-ER / GFP
α-Syn-DsRed / GFP
Mock
MTM6A12
E
RFP/GFP fluorescence intensity
2.5
2.0
1.5
1.0
0.5
0.0
DsRed-ER
α-syn-DsRed
Mock
MTM6A12

DISCUSSION

In this study, I implemented a gain-of-function screening strategy to identify receptors that can bind to α-synuclein and mediate the cell-to-cell transmission of α-synuclein. Compared to the loss-of-function screening, the gain-of-function genetic screening can be a powerful method to identify new genes sufficient to confer a particular cellular phenotype. Gain-of-function screening generally begins with an introduction of cDNA expression libraries into cells. As a result, gain-of-function screening identifies only the "most potent" phenotype-conferring genes. Gain-of-screenings using different cell-based assays and cDNA expression library have been successfully performed in our laboratory (Shim et al., 2012; Park et al., 2014; Jung et al., 2016).

The development of a screening method to isolate the α-synuclein-binding membrane protein was successfully performed. The goal of this study was to isolate the membrane receptor that plays a critical role of α-synuclein uptake and propagation. Since there was no assay to directly analyze the propagation of α-synuclein by a gain of function screening, I have primarily focused on finding new α-synuclein-binding proteins. In fact, the binding with α-synuclein is the first step and essential to mediate its

propagation. As designed, isolation of the membrane proteins binding to α-synuclein was the first step and characterization of the role in the cell-to-cell propagation of α-synuclein was the second step. Of course, the next step would be a characterization of this role of MTM6A12 in *in vivo* system, including animal model.

I have screened α-synuclein-binding proteins through a novel screening assay. I screened about 1,800 human cDNAs encoding transmembrane proteins and several putative positive clones were successfully found by the primary and secondary screenings. Among them, MTM6A12 was the most effective clone. MTM6A12 is expressed in neurons of the brain and also in endothelial cells. Interestingly, MTM6A12 shows specific interaction with α-synuclein, but not with Aβ and tau oligomers. Our findings suggest that MTM6A12 is a novel cell surface membrane protein that possibly mediates cellular uptake of α-synuclein and plays a critical role in the spreading of α-synuclein pathology.

The important question is how the internalized α-synuclein exhibits neurotoxicity in PD. α-Synuclein has disordered conformations and forms various toxic oligomeric species (Xin et al., 2015). MTM6A12 can contribute to pathology in a different way, depending on various α-synuclein species. MTM6A12 is a novel protein and its function is not much known. One possibility of how

MTM6A12 contributes to neurotoxicity is that oligomeric α-synuclein binds to MTM6A12 and transmits the neurotoxic signal into cells. Alternatively, internalized α-synuclein via MTM6A12 may also induce neuroinflammatory signaling or Ca^{2+} dysregulation in non-neuronal cells and synaptic dysfunction in neuronal cells (Kim et al., 2013; Luth et al., 2014; Panicker et al., 2019). α-Synuclein is known to promote mitochondria reactive oxygen species (ROS) generation and mitochondrial deficits in neurons (Hsu et al., 2000). MTM6A12 could enhance the ROS production in mitochondria following uptake of α-synuclein.

The limitation of this study is that all experiments were performed using *in vitro* cell culture system and by the overexpression of MTM6A12. It is very important to investigate the transmission of α-synuclein in MTM6A12 knockout cells but it has not been successful. More, *in vivo* experiments using an animal model and analysis of patients with Parkinson's disease would be necessary to confirm the association with MTM6A12 and Parkinson's disease.

REFERENCE

Badawy, S. M. M., Okada, T., Kajimoto, T., Hirase, M., Matovelo, S. A., Nakamura, S., . . . Nakamura, S.-i. (2018). Extracellular α-synuclein drives sphingosine 1-phosphate receptor subtype 1 out of lipid rafts, leading to impaired inhibitory G-protein signaling. *Journal of Biological Chemistry, 293*(21), 8208-8216.

Brás, J., Guerreiro, R., & Hardy, J. (2015). SnapShot: genetics of Parkinson's disease. *Cell, 160*(3), 570-570. e571.

Breydo, L., Wu, J. W., & Uversky, V. N. (2012). α-Synuclein misfolding and Parkinson's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1822*(2), 261-285.

Choi, Y. R., Cha, S.-H., Kang, S.-J., Kim, J.-B., Jou, I., & Park, S. M. (2018). Prion-like propagation of α-synuclein is regulated by the FcγRIIB-SHP-1/2 signaling pathway in neurons. *Cell reports, 22*(1), 136-148.

Damier, P., Hirsch, E., Agid, Y., & Graybiel, A. (1999). The substantia nigra of the human brain: II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain, 122*(8), 1437-1448.

Desplats, P., Lee, H.-J., Bae, E.-J., Patrick, C., Rockenstein, E., Crews, L., . . . Lee, S.-J. (2009). Inclusion formation and neuronal cell death through neuron-to-neuron transmission of α-synuclein. *Proceedings of the National Academy of Sciences, 106*(31), 13010-13015.

Gąssowska, M., Czapski, G. A., Pająk, B., Cieślik, M., Lenkiewicz, A. M., & Adamczyk, A. (2014). Extracellular α-synuclein leads to microtubule destabilization via GSK-3β-dependent Tau phosphorylation in PC12 cells. *PloS one, 9*(4), e94259.

Goedert, M., Clavaguera, F., & Tolnay, M. (2010). The propagation of prion-like protein inclusions in neurodegenerative diseases. *Trends in neurosciences, 33*(7), 317-325.

Gwon, Y., Kam, T.-I., Kim, S.-H., Song, S., Park, H., Lim, B., . . . Jung, Y.-K. (2018). TOM1 regulates neuronal accumulation of amyloid-β oligomers by FcγRIIb2 variant in Alzheimer's disease. *Journal of Neuroscience, 38*(42), 9001-9018.

Halliday, G. M., Holton, J. L., Revesz, T., & Dickson, D. W. (2011). Neuropathology underlying clinical variability in patients with synucleinopathies. *Acta neuropathologica, 122*(2), 187-204.

Holmes, B. B., DeVos, S. L., Kfoury, N., Li, M., Jacks, R., Yanamandra, K., . . . Bagchi, D. P. (2013). Heparan sulfate proteoglycans mediate internalization and propagation of specific proteopathic seeds. *Proceedings of the National Academy of Sciences, 110*(33), E3138-E3147.

Hsu, L. J., Sagara, Y., Arroyo, A., Rockenstein, E., Sisk, A., Mallory, M., . . . Masliah, E. (2000). α-Synuclein promotes mitochondrial deficit and oxidative stress. *The American journal of pathology, 157*(2), 401-410.

Jucker, M., & Walker, L. C. (2013). Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature, 501*(7465), 45.

Jung, S., Nah, J., Han, J., Choi, S. G., Kim, H., Park, J., . . . Jung, Y. K. (2016). Dual-specificity phosphatase 26 (DUSP 26) stimulates Aβ42 generation by promoting amyloid precursor protein axonal transport during hypoxia. *Journal of neurochemistry, 137*(5), 770-781.

Kam, T.-I., Song, S., Gwon, Y., Park, H., Yan, J.-J., Im, I., . . . Song, D.-K. (2013). FcγRIIb mediates amyloid-β neurotoxicity and memory impairment in Alzheimer's disease. *The Journal of clinical investigation, 123*(7), 2791-2802.

Kim, C., Ho, D.-H., Suk, J.-E., You, S., Michael, S., Kang, J., . . . Lee, H.-J. (2013). Neuron-released oligomeric α-synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. *Nature communications, 4*, 1562.

Kordower, J. H., Chu, Y., Hauser, R. A., Freeman, T. B., & Olanow, C. W. (2008). Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nature*

medicine, 14(5), 504.

Lee, H.-J., Suk, J.-E., Bae, E.-J., & Lee, S.-J. (2008). Clearance and deposition of extracellular α-synuclein aggregates in microglia. *Biochemical and biophysical research communications, 372*(3), 423-428.

Li, J.-Y., Englund, E., Holton, J. L., Soulet, D., Hagell, P., Lees, A. J., . . . Björklund, A. (2008). Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nature medicine, 14*(5), 501.

Loria, F., Vargas, J. Y., Bousset, L., Syan, S., Salles, A., Melki, R., & Zurzolo, C. (2017). α-Synuclein transfer between neurons and astrocytes indicates that astrocytes play a role in degradation rather than in spreading. *Acta neuropathologica, 134*(5), 789-808.

Luk, K. C., Kehm, V., Carroll, J., Zhang, B., O'Brien, P., Trojanowski, J. Q., & Lee, V. M.-Y. (2012). Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science, 338*(6109), 949-953.

Luth, E. S., Stavrovskaya, I. G., Bartels, T., Kristal, B. S., & Selkoe, D. J. (2014). Soluble, prefibrillar α-synuclein oligomers promote complex I-dependent, Ca2+-induced mitochondrial dysfunction. *Journal of Biological Chemistry, 289*(31), 21490-21507.

Mao, X., Ou, M. T., Karuppagounder, S. S., Kam, T.-I., Yin, X., Xiong, Y., . . . Shin, J.-H. (2016). Pathological α-synuclein transmission initiated by binding lymphocyte-activation gene 3. *Science, 353*(6307), aah3374.

McCann, H., Stevens, C. H., Cartwright, H., & Halliday, G. M. (2014). α-Synucleinopathy phenotypes. *Parkinsonism & related disorders, 20*, S62-S67.

Panicker, N., Sarkar, S., Harischandra, D. S., Neal, M., Kam, T.-I., Jin, H., . . . Samidurai, M. (2019). Fyn kinase regulates misfolded α-synuclein uptake and NLRP3 inflammasome activation in microglia. *Journal of Experimental Medicine,*

216(6), 1411-1430.

Park, S., Choi, S.-G., Yoo, S.-M., Son, J. H., & Jung, Y.-K. (2014). Choline dehydrogenase interacts with SQSTM1/p62 to recruit LC3 and stimulate mitophagy. *Autophagy, 10*(11), 1906-1920.

Pujols, J., Peña-Díaz, S., Conde-Giménez, M., Pinheiro, F., Navarro, S., Sancho, J., & Ventura, S. (2017). High-throughput screening methodology to identify alpha-synuclein aggregation inhibitors. *International journal of molecular sciences, 18*(3), 478.

Scott, D. A., Tabarean, I., Tang, Y., Cartier, A., Masliah, E., & Roy, S. (2010). A pathologic cascade leading to synaptic dysfunction in α-synuclein-induced neurodegeneration. *Journal of Neuroscience, 30*(24), 8083-8095.

Shim, S. M., Lee, W. J., Kim, Y., Chang, J. W., Song, S., & Jung, Y.-K. (2012). Role of S5b/PSMD5 in proteasome inhibition caused by TNF-α/NFκB in higher eukaryotes. *Cell reports, 2*(3), 603-615.

Stefanova, N., Fellner, L., Reindl, M., Masliah, E., Poewe, W., & Wenning, G. K. (2011). Toll-like receptor 4 promotes α-synuclein clearance and survival of nigral dopaminergic neurons. *The American journal of pathology, 179*(2), 954-963.

Surguchev, A. A., & Emamzadeh, F. N. (2019). Cell Responses to Extracellular α-Synuclein. *Molecules, 24*(2), 305.

Vaquer-Alicea, J., & Diamond, M. (2019). Propagation of Protein Aggregation in Neurodegenerative Diseases. *Annual review of biochemistry*.

Volpicelli-Daley, L. A., Luk, K. C., Patel, T. P., Tanik, S. A., Riddle, D. M., Stieber, A., . . . Lee, V. M.-Y. (2011). Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. *Neuron, 72*(1), 57-71.

Xin, W., Emadi, S., Williams, S., Liu, Q., Schulz, P., He, P., . . . Sierks,

M. (2015). Toxic oligomeric alpha-synuclein variants present in human Parkinson's disease brains are differentially generated in mammalian cell models. *Biomolecules, 5*(3), 1634-1651.

Yamada, K., & Iwatsubo, T. (2018). Extracellular α-synuclein levels are regulated by neuronal activity. *Molecular neurodegeneration, 13*(1), 9.

국문 초록

파킨슨 병은 운동 능력에 이상이 생기는 신경 퇴행성 질환이며 알파시뉴클레인 단백질 집합체의 축적이 신경 병리학 적으로 특징으로 나타난다. 알파시뉴클레인은 다양한 방법으로 파킨슨 병에 기여할 수 있다. 알파시뉴클레인 응집체가 신경 세포 사이를 이동하며 프리온과 같은 방식으로 신경 세포에 독성을 갖는 응집체를 형성할 수 있다는 연구들이 보고되었다. 그러나 알파시뉴클레인 응집체가 신경 세포 간 이동에 대한 메커니즘은 잘 알려져 있지 않다. 최근의 연구에서 LAG3 단백질이 알파시뉴클레인 응집체의 세포 내 유입을 매개하는 신경 세포 수용체로 처음 보고되었다. 또한, 알파시뉴클레인은 여러 가지 형태의 응집체를 형성하고 여러 종류의 막 단백질 수용체에 결합할 수 있다. 이 연구에서는 알파시뉴클레인의 세포 내 흡수를 매개하는 막 단백질 수용체를 찾기 위해 세포 기반 분석법을 확립하고 막 단백질을 발현시키는 인간 유전자 cDNA 라이브러리를 이 분석 방법을 사용하여 스크리닝하였다. 1 차 및 2 차 스크리닝에서 알파시뉴클레인 중합체와 결합을 증가시키는 여러 개의 후보 유전자를 찾아내었다. 이들 중 MTM6A12이 알파시뉴클레인 중합체와 강하게 결합하였다. MTM6A12의 과발현에 의해 알파시뉴클레인과 세포와의 결합이 증가하고, 알파시뉴클레인의 세포 내 유입이 알파시뉴클레인 처리 농도가 증가함에 따라 증가한다. 또한, MTM6A12는 아밀로이드 베타와 타우 중합체에는 결합하지 않고 알파시뉴클레인 중합체에 특이적으로 결합한다. 결과적으로 이 실험을 통해 파킨슨병의 병리학적 특징인 알파시뉴클레인의 세포 간 이동에 관여하는 새로운 막

단백질을 찾을 수 있었다.

주요어: 파킨슨병, 알파시뉴클레인, 시뉴클레인병증, 수용체, 세포 내 유입, 세포간 이동, 병리적 단백질의 전파

학번: 2017-24913